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ProcartaPlex™

Multiplex Immunoassay

Using Magnetic Beads
For Serum, Plasma, and Cell Culture Supernatant Samples

Instructions for
Human Isotyping Panel

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Citing ProcartaPlex™ Immunoassay in Publications

When describing a procedure for publication using this product, please refer to it as the ProcartaPlex™ Multiplex Immunoassay from eBioscience.

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Contents

Intended Use

This user manual is for a ProcartaPlex™ Human Isotyping 7plex Kit - Magnetic Beads from eBioscience to perform quantitative detection of Human IgG1, IgG2, IgG3, IgG4, IgA, IgM and IgE by using Luminex® technology. The procedure is for simultaneous measurements of multiple protein biomarkers in serum, plasma, and cell culture supernatant samples. Other biological samples might be suitable for use in the assay.

The assay protocol and reagents supplied are not compatible with other manufacturer's reagents. Each 96-Well Plate kit is configured to allow for the following usage: 14 wells for a 7-point standard curve (in duplicate), 2 wells for blanks, and up to 80 wells for samples.

NOTE: ProcartaPlex Human Antibody Isotyping Panel cannot be combined with other ProcartaPlex Simplex or Multiplex Kits.

NOTE: Simplex Kits can only be combined with analytes included in ProcartaPlex Human Isotyping Panel.

NOTE: For the most current version of user documentation, go to our website at www.ebioscience.com

Summary

Antibodies are soluble immunoglobulins (Ig) produced by plasma cells in response to an immunogen and are critically involved in the immune response. Each immunoglobulin actually binds to a specific antigenic determinant (epitope).

All immunoglobulins have a four chain structure as their basic unit. They are composed of two identical light chains (lambda or kappa; 23kD) and two identical heavy chains (alpha, delta, gamma, epsilon or mu; 50-70kD) connected by disulfide bonds.

Based on the differences in the amino acid sequences in the constant region (Fc-part) of the heavy chains the immunoglobulins can be divided into five different classes: IgG, IgM, IgA, IgD and IgE.

IgGs can additionally be divided into 4 subclasses based on the number of disulfide bonds and length of the hinge region.

If different immunoglobulin classes or isotypes recognize an antigen at the same epitope, they still differ in the way the immune system reacts to the antigen. The specific Fc-part will promote a specific effector function of the immune system to the antigen.

The isotype of an antibody determines its distribution to the different compartments of the body in which their particular effector functions are appropriate.

How it Works

ProcartaPlex™ Immunoassays use the xMAP® technology (multi-analyte profiling beads) to enable the detection and quantitation of multiple protein targets simultaneously in diverse matrices. The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), dual laser design and digital signal processing to effectively allow multiplexing of up to 100 (50 for MAGPIX) unique assays within a single sample. The ProcartaPlex Immunoassay Kits are compatible with all Luminex and Luminex-based instruments currently available.

Analyte Overview

All products listed on our website www.eBioscience.com

ProcartaPlex Workflow

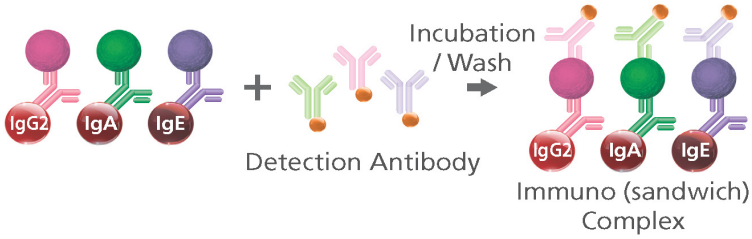
Figure 1

Capture Target Analytes

- Add analyte-specific capture beads coated with target-specific capture antibodies to prepared sample.

Incubate 1-2 hours and wash

Figure 2

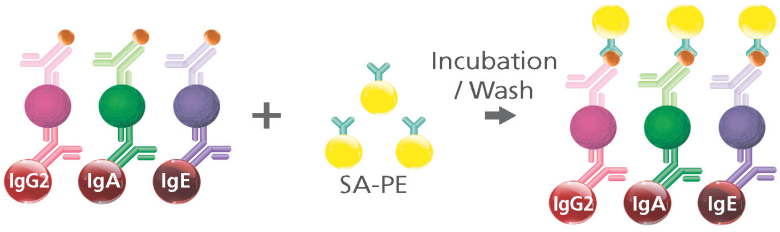


Detect Captured Analyte

- Incubate captured bead/analytes with biotinylated analyte-specific detection

Incubate 30 min and wash

Figure 3



Label Detection

- For analyte quantitation, incubate with a fluorescent detection label (SA-PE)
- Wash sample
- Quantitate sample

Incubate 30 min and wash

Figure 4

Read & Analyze

- Read with Luminex Instrument
- Analyze results with ProcartaPlex Analyst Software 1.0

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Free download of ProcartaPlex Analyst Software 1.0 at: ebioscience.com/ppx-analyst

Components

ProcartaPlex Immunoassay Kits contain the components listed below. Refer to the Certificate of Analysis included in the kit for quantities and details of components supplied.

Expiration of the kit is stated on the kit label. Expiration of the kit components can only be guaranteed if the components are stored properly between 2-8°C, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

Component	Description
Antigen Standards, premixed, lyophilized (2 vials each set)	Please note that more than 1 set of vials may be shipped with each kit for certain products
Detection Antibody, premixed (50X) [1]	Concentrated biotinylated detection antibodies
Antibody Magnetic Beads, premixed[1]	Magnetic beads coated with specific antibodies
Streptavidin-PE (SA-PE)[1]	Streptavidin-conjugated R-phycoerythrin
Wash Buffer Concentrate (10X)[1]	Concentrated aqueous buffered solution
Detection Antibody Diluent[1]	For dilution of detection antibodies
Universal Assay Buffer Concentrate (10X)[1]	For analytes with high serum and plasma concentration additional UAB (10X) will be included
Reading Buffer[1]	Aqueous buffered solution
PCR 8-Tube Strip (2X)	0.2 mL 8-Tube Strip for preparing standard curve
Filter Plate	96-Well Filter Plate for use with a vacuum system
Flat Bottom Plate	96-Well Flat Bottom Plate for use with hand held magnet
Black Microplate Lid	Protecting the assay from light during incubation
Plate Seals	Adhesive-backed foil plate sealer

[1])Contains Sodium Azide. See WARNING on next page.

WARNING: All chemicals should be considered potentially hazardous. We recommend that this product and its components be handled by those trained in laboratory techniques and be used according to the principles of good laboratory practice.

WARNING: This kit contains small quantities of sodium azide. Sodium azide is highly toxic and reactive in the pure form. At this product's concentration, though not classified as hazardous, build up of sodium azide may react with lead and copper plumbing to form highly reactive explosive metal azide. Dispose of the product in accordance with all state and local regulations.

Required Equipment and Materials Not Supplied

- Luminex® 100/200, MAGPIX®, FlexMap® 3D, or other Luminex-based Instrument.
- Glass-distilled or deionized water.
- 5 mL and 10 mL graduated pipettes.
- 10 -1000 µL adjustable single channel micropipettes with disposable tips.
- 20 µL to 300 µL adjustable multichannel micropipettes with disposable tips.
- Multichannel micropipette reservoir.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- Vortex mixer and Microplate shaker.
- Hand-Held Magnetic Plate Washer, Vacuum Filtration Manifold.

Precautions and Technical Hints

- Thoroughly read this user manual and product insert that is included with the assay kit. The product insert may contain specific instructions for proper use of your kit.
- Before starting the assay, turn on the Luminex machine and initiate the startup protocol. It takes 30 min for the lasers to warm-up. Make sure the Luminex machine is calibrated according to the manufacturer's instructions.
- When working with samples and standards, change the pipette tips after every transfer and avoid creating bubbles when pipetting.
- Some samples may contain high analyte concentrations and require sample dilution for accurate quantitation. Please use cell culture medium for cell culture supernatants or Universal Assay Buffer for serum and plasma samples.
- During the incubation steps, cover the 96-Well Plate with the Black Microplate Lid provided in the kit to minimize exposure of the beads to light.
- Be careful not to invert the ProcartaPlex 96-Well Flat Bottom Plate during the assay or allow contents from one well to mix with another well.
- Ensure that the Hand-Held Magnetic Plate Washer is securely locked into place prior to inverting when performing the wash steps.
- Use a multi-channel pipette and reagent reservoirs whenever possible to achieve optimal assay precision.
- Store the Detection Antibody, Antibody Magnetic Beads, Streptavidin-PE, samples, and reconstituted standards (including standard diluent sets) on ice before adding to the 96-Well Plate.
- For frozen samples, thaw completely on ice and mix well prior to running the assay.

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice (www.eBioscience.com).
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugates.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Decontaminate and dispose of specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

Sample Preparation

Cell culture supernatant, serum, and plasma samples have been tested and validated with the ProcartaPlex assay. Although not validated, other biological samples such as BAL, saliva, CSF, tissue lysates, and urine might be suitable for use in the assay.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly lipemic specimens. Fat causes agglutination of the beads. Centrifugation of samples (about 16,000 *x g* for 5 min) before analysis is recommended.

For serum or plasma samples, remove the clot or cells as soon as possible after clotting and separation. A total volume of 25 µL per well of serum and plasma or 50 µL per well of cell culture supernatant samples is needed and a minimum of 2 replicates is recommended.

The analytes included in the kit have high serum or plasma concentrations.

1:20.000-fold dilution in Universal Assay Buffer (1x) is appropriate for most serum and plasma samples. For samples that yield results outside the range of the standard curve, a lower or higher dilution might be required. For cell culture supernatant, a 1:200 dilution is suggested.

Each serum or plasma sample has to be diluted **1:20,000** according to following scheme:

Dilution 1 (1:200): 20 µl sample + 1980 µl Universal Assay Buffer (1x)

Dilution 2 (1:100): 10 µl prediluted sample (1:200)+ 990 µl Universal Assay Buffer (1x)

If the samples are taken from cell culture supernatant prepare a **1:200** dilution:

Dilution 1 (1:10): 10 µl sample + 90 µl cell culture medium (1x)

Dilution 1 (1:20): 50µl prediluted sample (1:10) + 950 µl cell culture medium (1x)

NOTE: Cell culture supernatant concentrations are cell-line dependent and range from 5-50 µg/mL. Bioreactor supernatants may be as concentrated as 1 mg/mL and thus need higher dilutions.

NOTE: For analytes with high serum and plasma concentration additional Universal Assay Buffer (10X) will be included in the kit.

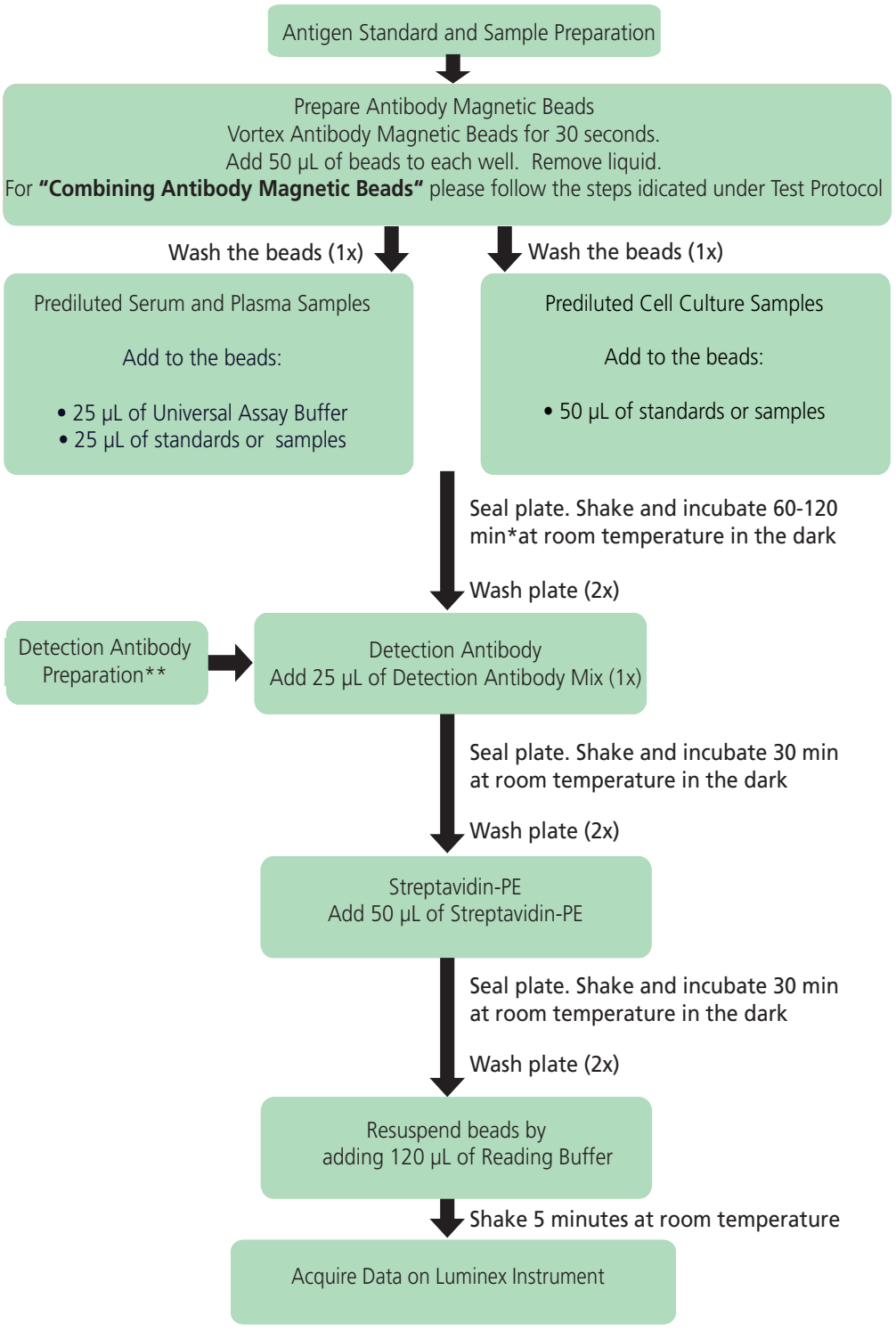
Preparing Plasma Samples

1. Collect samples in sodium citrate or EDTA tubes. When using heparin as an anticoagulant, no more than 10 IU of heparin per mL of blood collected should be used since an excess of heparin may give falsely high values of some of the analytes.
2. Centrifuge samples at 1,000 *x g* at 4 °C for 10 min within 30 min of blood collection.
3. Collect the plasma fraction.
4. (Optional) To minimize lipid and/or platelets in the sample, centrifuge the sample at 10,000 *x g* for 10 min at 2-8 °C and collect the plasma fraction.
5. Use immediately or aliquot and store below -20 °C.

Preparing Serum Samples

1. Allow blood to clot for 20-30 min at 20-25 °C.
2. Centrifuge at 1,000 *x g* for 10 min at 20-25 °C.
3. Collect the serum fraction. (Alternatively, use any standard serum separator tube following the manufacturer's instructions.)
4. (Optional) If there is a high lipid content in the sample, centrifuge at 10,000 *x g* for 10 min at 2-8 °C. Collect the serum fraction.
5. Use immediately or aliquot and store below -20 °C.

Assay Protocol Overview



*For assays that require higher sensitivity, 120 min or overnight incubation at 4°C is recommended.
**Concentration of Detection Antibody in Customized Kits is 1x, therefore no further dilution is needed.

Preparation of Reagents

Wash Buffer

Wash Buffer Concentrate (10X) should be brought to room temperature and should be diluted before starting the test procedure. If crystals have formed in the Wash Buffer Concentrates (10X), warm them gently until they have completely dissolved.

1. Dilute the Wash Buffer Concentrate (10X) 1:10 with deionized water. E.g. mix 20mL of the Wash Buffer Concentrate (10X) with 180 mL deionized water.
2. Store the Wash Buffer (1X) at 2-8°C. The Wash Buffer is stable for 6 months, when stored properly.

Universal Assay Buffer (10X)

Dilute the Universal Assay Buffer Concentrate (10X) with deionized water.

E.g. mix 10 mL of Universal Assay Buffer Concentrate (10X) with 90 mL deionized water.

Store the Universal Assay Buffer Concentrate (10X) at 2° to 8°C. When stored properly the Universal Assay Buffer (1X) is stable for 30 days.

Standard

This section provides instructions on how to make a 7-point standard curve for the assay panel. Each kit is shipped with two vials of identical premixed antigen standards. Please refer to the Certificate of Analysis provided in the kit for the content of analytes in each premixed standard and when assigning standard antigen concentrations for each analyte.

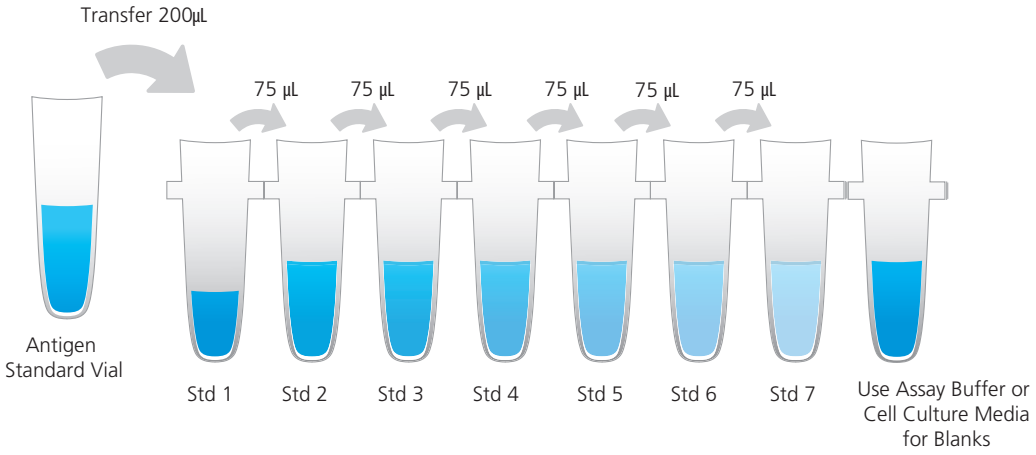
NOTE: Standard Range vary from lot to lot. The Certificate of Analysis (CoA) includes the S1 data of your kit.

For antigen standard preparation follow the steps indicated below:

1. Centrifuge the antigen standard vial at 2000 x g for 10 sec.
2. Add 250 µL of sample type-specific standard buffer (for serum or plasma use Universal Assay Buffer; for cell culture supernatants use cell culture medium) into the vial.
3. Vortex gently for 30 sec.
4. Incubate on ice for 5-10 min.

Preparing a 3-Fold Serial Dilution

1. Prepare a 3-fold serial dilution of the standard mix (reconstituted according to the text above) using the PCR 8-Tube Strip provided.
2. Add 200 µL of the reconstituted antigen standard mix into the first strip tube and label the tube as Standard 1 (Std 1).
3. Add 150 µL sample type-specific standard buffer (for serum or plasma use Universal Assay Buffer; for cell culture use cell culture medium) into tubes 2-7 and label them accordingly.
4. Transfer 75 µL of the reconstituted antigen standards from tube 1 into tube 2.
5. Mix by pipetting up and down for a total of 10 times.
6. After changing the pipette tip, transfer 75 µL of the mixed standards from tube 2 into tube 3.
7. Mix by pipetting up and down 10 times.
8. Repeat actions 4 to 7 for the rest of the tubes to prepare Std 4-7. Add Universal Assay Buffer or cell culture medium into tube 8 that serves as blank. Keep on ice until ready to use.



Detection Antibody Mixture (1X)

Detection Antibodies in Simplex Kits and Panels are provided as 50X concentrate. Some larger plexed panels include more than one premixed Detection Antibody Concentrate in the kit. If you want to include additional simplex assays in your multiplex assay, the Detection Antibody Concentrate provided in the Simplex Kits must also be included in the Detection Antibody Mixture (1X).

If your kit includes Detection Antibody Concentrate (50X) prepare the Detection Antibody Mixture (1X) according to the following calculation:

1. Each well uses 25 µL of the Working Detection Antibody Mixture (1X). Prepare an appropriate volume of Working Detection Antibody Mixture (1X) by combining the concentrated Detection Antibody (50X) with the Detection Antibody Diluent.
2. Scale according to the number of assays to be run, and include sufficient overage. Use the table below as a guide. When calculating the number of wells, make sure to consider the total number of wells needed that include the standard curves, blanks and samples. Calculate the final volume of the Detection Antibody Mixture (1X) needed and estimate for 25% overage to enable use of a reagent reservoir and multi-channel pipette

Example for 1 vial of Detection Antibody

	24 Well	48 Wells	96 Wells
Detection Antibody	15 µL	30 µL	60 µL
Detection Antibody Diluent	735 µL	1470 µL	2940 µL
Total	750 µL	1500 µL	3000 µL

Example for 2 vials of Detection Antibodies

	24 Well	48 Wells	96 Wells
Detection Antibody 1	15 µL	30 µL	60 µL
Detection Antibody 2	15 µL	30 µL	60 µL
Detection Antibody Diluent	720µL	1440 µL	2880 µL
Total	750 µL	1500 µL	3000 µL

Protocol using a Hand-Held Magnetic Plate Washer

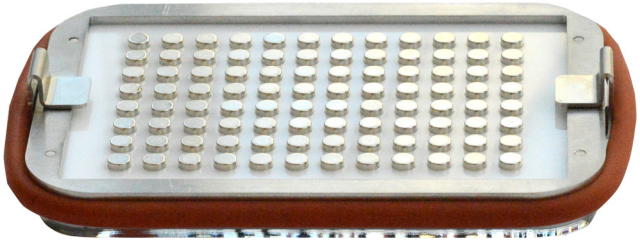
Prepare the 96-Well Flat Bottom Plate

For your convenience please use the blank layout provided in the Layout section to define your plate scheme (blanks, standards and samples).

Add Antibody Magnetic Beads

If only **one** Antibody Magnetic Bead Mixture is included in the kit, please follow the steps indicated below:

1. Vortex the Antibody Magnetic Beads for 30 sec.
2. Add 50 µL of the Antibody Magnetic Beads to each well.
3. Insert the ProcartaPlex 96-Well Flat Bottom Plate into the Hand-Held Magnetic Plate Washer.



4. Place the 96-Well Plate by pushing the 2 securing tabs, located on each end of the washer. Rubber gasket surrounds the system and provides optimal mount of the 96-Well Flat Bottom Plate.
5. Verify that the 96-Well Flat Bottom Plate is securely locked by holding the assembly in the palm of your hand and gently pulling up on the 96-Well Flat Bottom Plate.
6. Wait 2 min to allow the Antibody Magnetic Beads to accumulate on the bottom of each well.
7. Remove the liquid in the wells by quickly inverting the Hand-Held Magnetic Plate Washer and 96-Well Flat Bottom Plate assembly over a sink or waste container. Do not remove the 96-Well Flat Bottom Plate from the Hand-Held Magnetic Plate Washer. Blot the inverted assembly onto several layers of paper towels to remove any residual solution.
8. Proceed to the step **"Wash Antibody Magnetic Beads using a Hand-Held Magnetic Plate Washer"**.

Combining Antibody Magnetic Beads from multiple kits

Multiple Simplex Kits can be combined with one another as well as with Premixed Panels. When mixing two or more Simplex Kits or Premixed Panels, ensure that the bead regions assigned to the analytes do not overlap. Some analytes use the same bead region and thus cannot be combined together in one multiplex assay. Please check the compatibility of analytes online (Panel Configurator) or contact your local sale representative.

Both Simplex and Premixed Kits are supplied with beads at working concentration. Our test protocol requires 50 µL beads per well. The 96-Well Flat Bottom Plate used can hold up to 300 µL per well, so 6 different Antibody Magnetic Beads can be premixed at one time using the procedure below for use with the Hand-Held Magnetic Washer. If more than 6 bead mixtures are to be mixed, then this procedure can be repeated.

If **two** or **more** Antibody Magnetic Bead Mixtures are included in the kit, please follow the steps indicated below (for 96-wells):

1. Place the 96-Well Flat Bottom Plate onto the Hand-Held Magnetic Washer and securely lock into place.
2. Vortex each of the Antibody Magnetic Beads for 30 sec and add 5 mL of each Bead Mixture to an appropriate vial.
3. Vortex the Antibody Magnetic Beads for 30 sec.
4. Add the appropriate volume of Antibody Magnetic Beads to each well.
   - 100 µL to each well if 2 different Antibody Magnetic Beads have been mixed
   - 150 µL to each well if 3 different Antibody Magnetic Beads have been mixed
   - 200 µL to each well if 4 different Antibody Magnetic Beads have been mixed
   - 250 µL to each well if 5 different Antibody Magnetic Beads have been mixed
   - 300 µL to each well if 6 different Antibody Magnetic Beads have been mixed
5. Wait 2 min to allow the Antibody Magnetic Beads to accumulate on the bottom of each well.
6. Remove the liquid in the wells by quickly inverting the Hand-Held Magnetic Plate Washer and 96-Well Flat Bottom Plate assembly over a sink or waste container. Blot the inverted assembly onto several layers of paper towels to remove any residual solution.
7. Leave 96-Well Flat Bottom Plate on Hand-Held Magnetic Plate Washer and proceed to the step **“Wash Antibody Magnetic Beads using a Hand-Held Magnetic Plate Washer”** on page 18.

If a suitable tube **Magnetic Bead Separator** is available, premix the beads using the following protocol:

1. Vortex the Antibody Magnetic Beads for 30 sec.

2. Add 5 mL for 96-wells of each Bead Mixture to an appropriate vial.
3. Let the beads accumulate according to the instructions of the separator.
4. Remove the liquid. If necessary, repeat 1-4 until all beads are in the tube.
5. Place the 96-Well Flat Bottom Plate onto the Hand-Held Magnetic Washer and securely lock into place.
6. If using the whole plate (96-wells) re-suspend the beads in 16 mL Wash Buffer. Vortex the Antibody Magnetic Beads for 30 sec and add 150 µL of the Antibody Magnetic Beads to each well of the plate.
7. Wait 2 min to allow the Antibody Magnetic Beads to accumulate on the bottom of each well.
8. Remove the liquid in the wells by quickly inverting the Hand-Held Magnetic Plate Washer and 96-Well Flat Bottom Plate assembly over a sink or waste container. Blot the inverted assembly onto several layers of paper towels to remove any residual solution.
9. Proceed to the step **"Add Standards and Samples".**

Wash Antibody Magnetic Beads using a Hand Held Magnetic Plate Washer

1. Add 150 µL of 1X Wash Buffer into each well.
2. Wait 30 sec to allow the Antibody Magnetic Beads to accumulate on the bottom of each well.
3. Remove the Wash Buffer in the wells by quickly inverting the Hand-Held Magnetic Washer and 96-Well Flat Bottom Plate assembly over a sink or waste container.
4. Blot the inverted assembly onto several layers of paper towels to remove any residual solution.

Add Standards and Samples

Serum and Plasma Samples

1. Add 25 µL of Universal Assay Buffer into each well.
2. Add 25 µL of standards or samples into dedicated wells.
3. Add 25 µL of Universal Assay Buffer to the blank wells.

Cell Culture Supernatant Samples

1. Add 50 µL of standards or samples into dedicated wells.
2. Add 50 µL of cell culture medium to the blank wells.

Incubate the 96-Well Flat Bottom Plate

1. Seal the 96-Well Flat Bottom Plate using a Plate Seal provided.
2. Remove the 96-Well Flat Bottom Plate from the Hand-Held Magnetic Plate Washer and cover the plate with the Black Microplate Lid provided in the kit in order to protect it from light.
3. During incubation shake the 96-Well Flat Bottom Plate at 500 rpm for 60 min at room temperature.

Wash the 96-Well Plate using a Hand-Held Magnetic Plate Washer

1. Insert the 96-Well Flat Bottom Plate into the Hand-Held Magnetic Plate Washer and wait 2 min to allow the Antibody Magnetic Beads to accumulate on the bottom of each well.
2. Carefully remove the Plate Seal to avoid splashing the plate contents.
3. Remove the solution in the wells by quickly inverting the Hand-Held Magnetic Plate Washer and 96-Well Flat Bottom Plate assembly over a sink or waste container.
4. Add 150 µL of 1X Wash Buffer into each well.
5. Wait 30 sec to allow the Antibody Magnetic Beads to accumulate on the bottom of each well.
6. Remove the supernatant in the wells by quickly inverting the 96-Well Plate over a sink or waste container. Repeat actions 4-6 one more time for a total of 2 washes.
7. After the last wash, blot the assembly onto several layers of paper towels to remove any residual solution.

NOTE: When washing the 96-Well Plate, we recommend using a multi-channel pipette or a multi-channel automatic liquid dispenser. Avoid touching the pipette tips to the sides of the wells when adding Wash Buffer using a multi-channel pipette.

Add Detection Antibodies Mixture (1X)

1. Add 25 µL of Detection Antibodies Mixture (1X) into each well.
2. Seal the 96-Well Plate with a new Plate Seal.
3. Remove the 96-Well Plate from the Hand-Held Magnetic Plate Washer and cover the plate with the Black Microplate Lid provided in the kit.
4. Shake at 500 rpm for 30 min at room temperature.

Wash the 96-Well Flat Bottom Plate

Repeat steps indicated under **“Wash the 96-Well Plate using a Hand-Held Magnetic Plate Washer”.**

Add Streptavidin-PE (SA-PE)

1. Add 50 µL of SA-PE solution into each well.
2. Seal the 96-Well Plate with a new Plate Seal.
3. Remove the 96-Well Plate from the Hand-Held Magnetic Plate Washer and cover the plate with the Black Microplate Lid provided in the kit.
4. Shake at 500 rpm for 30 min at room temperature.

Wash the 96-Well Flat Bottom Plate

Repeat steps indicated under **"Wash the 96-Well Plate using a Hand-Held Magnetic Plate Washer".**

Prepare the Plate for Analysis on a Luminex Instrument

1. Add 120 µL of Reading Buffer into each well.
2. Seal the 96-Well Plate with a new Plate Seal.
3. Remove the 96-Well Plate from the Hand-Held Magnetic Plate Washer and cover the plate with the Black Microplate Lid provided in the kit.
4. Shake at 500 rpm for 5 min at room temperature.
5. Remove the Plate Seal prior to reading on the Luminex Instrument.

Protocol using a Filter Plate

The Antibody Magnetic Beads can be used with a Vacuum Filtration Manifold and the 96-Well Filter Plate. The Vacuum Manifold can be properly calibrated by placing a standard 96-Well Filter Plate on top of the manifold and then turning on the vacuum. Press down on all 4 corners of the standard 96-Well Filter Plate to form a tight seal and adjust the pressure so that it takes 4-6 seconds to evacuate 150 µL of Wash Buffer from the wells. If the vacuum is too high, beads can get trapped or pulled through the filter. Turn off vacuum as soon as the solution filters through the wells and remove the plate from the manifold.

Prepare the 96-Well Filter Plate

1. For your convenience please use the blank layout provided in the Layout section to define your plate scheme (blanks, standards and samples).
2. Add 50 µL Reading Buffer (1X) to the filter plate to pre-wet the wells. Aspirate using the Vacuum Filtration Manifold. Blot the bottom of the plate after filtration.

Add Antibody Magnetic Beads

If only **one** Antibody Magnetic Bead Mixture is included in the kit, please follow the steps indicated below:

1. Vortex the Antibody Magnetic Beads for 30 sec.
2. Add 50 µL of the Antibody Magnetic Beads to each well.
3. Remove the liquid in the wells by aspiration using the Vacuum Filtration Manifold. Blot the bottom of the plate after filtration.
4. Proceed to the step **"Wash Antibody Magnetic Beads using a 96-Well Filter Plate".**

Combining Antibody Magnetic Beads from multiple kits

Multiple Simplex Kits can be combined with one another as well as with Premixed Panels. When mixing two or more Simplex Kits or Premixed Panels, ensure that the bead regions assigned to the analytes do not overlap. Some analytes use the same bead region and thus cannot be combined together in one multiplex assay. Please check the compatibility of analytes online (Panel Configurator) or contact your local sale representative.

Both Simplex and Premixed Kits are supplied with beads at working concentration.Our test protocol requires of 50 µL beads per well. The 96-Well Filter Plate used can hold up to 300 µl per well so 6 different Antibody

Magnetic Beads can be premixed at one time using the procedure below for use with the Vacuum Filtration Manifold. If more than 6 bead mixtures are to be mixed, then this procedure can be repeated.

If **two** or **more** Antibody Magnetic Bead Mixtures are included in the kit, please follow the steps indicated below:

1. Vortex the Antibody Magnetic Beads for 30 sec and add 5 mL for 96-wells of each Bead Mixture to an appropriate vial.
2. Vortex the Antibody Magnetic Beads for 30 sec.
3. Add the appropriate volume of Antibody Magnetic Beads to each well.
   - 100 µL to each well if 2 different Antibody Magnetic Beads have been mixed
   - 150 µL to each well if 3 different Antibody Magnetic Beads have been mixed
   - 200 µL to each well if 4 different Antibody Magnetic Beads have been mixed
   - 250 µL to each well if 5 different Antibody Magnetic Beads have been mixed
   - 300 µL to each well if 6 different Antibody Magnetic Beads have been mixed
4. Remove the liquid in the wells by aspiration using the Vacuum Filtration Manifold. Blot the bottom of the plate after filtration.
5. Proceed to the step **"Wash Antibody Magnetic Beads using a 96-Well Filter Plate"** below.

If a suitable tube **Magnetic Bead Separator** is available, premix the beads using the following protocol:

1. Vortex the Antibody Magnetic Beads for 30 sec.
2. Add 5 mL for 96-wells of each Bead Mixture to an appropriate vial.
3. Let the beads accumulate according to the instructions of the separator.
4. Remove the liquid. If necessary, repeat 1-4 until all beads are in the tube.
5. If using the whole plate (96-Wells) re-suspend the beads in 16 mL Wash Buffer. Vortex the Antibody Magnetic Beads for 30 sec and add 150 µL of the Antibody Magnetic Beads to each well of the plate.
6. Remove the liquid in the wells by aspiration using the Vacuum Filtration Manifold. Blot the bottom of the plate after filtration.
7. Proceed to the step **"Add Standards and Samples".**

Wash Antibody Magnetic Beads using a 96-Well Filter Plate

1. Add 150 µL of 1X Wash Buffer into each well.
2. Remove the liquid in the wells by aspiration using the Vacuum Filtration Manifold. Blot the bottom of the plate after filtration.

Add Standards and Samples

Serum and Plasma Samples

1. Add 25 µL of Universal Assay Buffer into each well.
2. Add 25 µL of standards or samples into dedicated wells.
3. Add 25 µL of Universal Assay Buffer to the blank wells.

Cell Culture Supernatant Samples

1. Add 50 µL of standards or samples into dedicated wells.
2. Add 50 µL of cell culture medium to the blank wells.

Incubate the 96-Well Filter Plate

1. Seal the 96-Well Filter Plate using a Plate Seal provided.
2. Cover the plate with the Black Microplate Lid provided in the kit.
3. During incubation shake the 96-Well Filter Plate at 500 rpm for 60 to 120 min at room temperature.

Wash the 96-Well Filter Plate

1. Carefully remove the Plate Seal to avoid splashing the plate contents.
2. Remove the liquid in the wells by aspiration using the Vacuum Filtration Manifold. Blot the bottom of the plate after filtration.
3. Add 150 µL of 1X Wash Buffer into each well.
4. Remove the liquid in the wells by aspiration using the Vacuum Filtration Manifold. Blot the bottom of the plate after filtration.
5. Repeat actions 3-4 one more time for a total of 2 washes.

NOTE: When washing the 96-Well Plate, we recommend using a multi-channel pipette or a multi-channel automatic liquid dispenser. Avoid touching the pipette tips to the sides of the wells when adding Wash Buffer using a multi-channel pipette.

Add Detection Antibodies Mixture (1X)

1. Add 25 µL of Detection Antibodies Mixture (1X) into each well.
2. Seal the 96-Well Filter Plate with a new Plate Seal and cover the plate with the Black Microplate Lid provided in the kit.

3. Shake at 500 rpm for 30 min at room temperature.

Wash the 96-Well Filter Plate

Repeat steps indicated under **"Wash the 96-Well Filter Plate".**

Add Streptavidin-PE (SA-PE)

1. Add 50 µL of SA-PE solution into each well.
2. Seal the 96-Well Filter Plate with a new Plate Seal and cover the plate with the Black Microplate Lid provided in the kit.
3. Shake at 500 rpm for 30 min at room temperature.

Wash the 96-Well Filter Plate

Repeat steps indicated under **"Wash the 96-Well Filter Plate"**.

Prepare the Plate for Analysis on a Luminex Instrument

1. Add 120 µL of Reading Buffer into each well.
2. Seal the 96-Well Filter Plate with a new Plate Seal.
3. Cover the plate with the Black Microplate Lid provided in the kit.
4. Shake at 500 rpm for 5 min at room temperature.
5. Remove the Plate Seal prior to reading on the Luminex Instrument.

Setup of the Luminex Instrument

Sample Size	DD Gate	Timeout	Bead Event/Bead Region
50 µL	5,000 - 25,000	45 sec	50-100

If you are running assays on your Luminex Instrument that uses both the 96-Well Flat Bottom Plate and 96-Well Filter Plate for Antibody Magnetic Beads verify the probe height for each plate type before reading. Failure to adjust the probe height can cause damage to the instrument.

The Luminex system allows for calibration of Low and High RP1 target values. We recommend RP1 Low target value settings for ProcartaPlex Immunoassays.

Please refer to the Certificate of Analysis provided with the kit for bead region and analyte associations when entering the information into the Luminex Acquisition Software.

NOTE: **If there is a malfunction of the Luminex Instrument or software during the run, the 96-Well Plate can be re-read. Remove the 96-Well Plate from the instrument, insert the 96-Well Plate into the Hand-Held Magnetic Plate Washer, wait 2 min, then remove the buffer in the wells by quickly inverting the 96-Well Plate over a sink or waste container. Blot the assembly onto several layers of paper towels to remove any residual solution. Resuspend the beads in 120 µL of Reading Buffer, remove from the Hand-Held Magnetic Plate Washer, seal the 96-Well Plate with a new Plate Seal and shake at 500 rpm for 5 min at room temperature. The assayed samples may take longer to read since there will be less beads in the wells.**

NOTE: **If there is a malfunction of the Luminex Instrument or software during the run, the 96-Well Plate can be re-read. Remove the 96-Well Plate from the instrument and remove the liquid in the wells by aspiration using the Vacuum Filtration Manifold. Blot the bottom of the plate after filtration. Resuspend the beads in 120 µL of Reading Buffer, seal the 96-Well Plate with a new Plate Seal and shake at 500 rpm for 5 min at room temperature. The assayed samples may take longer to read since there will be less beads in the wells.**

Analyzing Results

The concentration of the samples can be calculated by plotting the expected concentration of the standards against the MFI generated by each standard. A 4PL or 5PL algorithm is recommended for the best curve fit. Analyze the assayed samples according to the operation manual for the Luminex, MAGPIX or Luminex-based Instrument. For reliable and quick analysis of the data ProcartaPlex Analyst 1.0 can be used.

NOTE: **Dilutions factors of samples have to be entered manually as described in the ProcartaPlex Analyst 1.0 Software Manual.**

Free download at: ebioscience.com/ppx-analyst

Specificity

Cross reactivity was tested with combinable analytes of Simplex and Multiplex ProcartaPlex Assays. There was no relevant cross reactivity observed. For detailed information refer to "Panel Configurator" on www.eBioscience.com.

Troubleshooting

Observation	Probable Cause	Recommend Solution
Low Flow Rate	Partial Blockage of the flow cell	Remove the 96-Well Plate and perform a wash and rinse cycle.
	Instrument needle is partially clogged	Replace or clean needle according to the manufacturer's recommendations.
High CVs	Contamination from re-using the Plate Seal	Use a new Plate Seal for each incubation step.
	Incomplete washing	Blot the 96-Well Plate onto several layers of paper towels to remove any residual solution after each wash step.
	Contamination from contents from adjacent wells	Avoid splashing the Wash Buffer during wash steps into adjacent wells.
	Poor pipetting techniques	Use appropriate pipetting techniques. Use new pipette tips for each well during sample and standard addition. Avoid touching pipette tips to sides of the wells when adding Wash Buffer.
Low bead count	Probe height is incorrect	Refer to the Luminex Manual for proper adjustment of the needle height.
	Reading buffer volume added in the last step to resuspend the beads is too low	Add 120 µL Reading Buffer into each well and shake at 500 rpm for 5 min at room temperature to resuspend the beads prior to reading on the Luminex Instrument. Make sure sample size is set at 100 µL in the acquisition protocol.
	High bead aggregation	Vortex the bead suspension well before using in the assay and ensure that the beads are properly mixed during the incubation steps.
		Verify that beads were added at the correct concentration, and that correct bead regions and wells were selected during acquisition setup.
	Dyes contained in the beads are photo-bleached from overexposure to light. Beads are falling outside the bead region gates due to photo bleaching.	Store bead solution in the dark and protect the 96-Well Plate from light by wrapping the 96-Well Plate with aluminum foil.
	Partial blockage of the flow cell	Remove the 96-Well Plate and perform a wash and rinse to the instrument.
	Instrument needle is partially clogged	Replace or clean needle according to the manufacturer's recommendations.
	Beads settle on the bottom of the well	Confirm that the plate shaker is set to 500 rpm and shaking for at least 5 min before reading.

Observation	Probable Cause	Recommend Solution
	Partial blockage of the flow cell	Remove the 96-Well Plate and perform a wash and rinse to the instrument. Clear system of clogs or air using maintenance steps described in the instrument user manual (sanitize, alcohol flush, probe sonication, etc.). Make sure that the probe height is set correctly. Make sure that beads are in suspension by shaking the plate for 3-5 minutes at 500 rpm immediately before analysis. Microbial growth in buffers can cause beads to stick to the filter plate membrane. Do not use contaminated reagents.
	Beads were exposed to organic solvents	Do not use organic solvents in the immunoassay, as they will damage beads irreversibly.
	Did not use supplied 96-well microtiter plate	Only use the ProcartaPlex 96-Well Flat Bottom Plate supplied with the kit.
	Air bubble in the sample loop	Refer to the Luminex Manual for proper removal of the air bubble.
Sample measurements not falling on the standard curve	Dilution of sample is too low or too high	If values are higher than the standard curve, dilute samples further in appropriate Sample Diluent.
	Target concentration is below detection	Verify that curve fitting at the lower end of the standard curve is accurate. Not all serum/plasma samples contain detectable levels of all analytes.
Low signal or sensitivity	Standards not reconstituted and diluted correctly	Prepare fresh antigen standards following the instructions in the Preparing Antigen Standards section.
	Expired reagents were used	Expiry of the kit and reagents is stated on labels. Do not use expired reagents.
	Suboptimal assay conditions	Follow the recommended incubation times and temperature. Shake the 96- Well Plate during all incubations except during optional overnight incubation step.
Poor accuracy	Did not use the appropriate assay diluents	Use the same sample type-specific standard and assay buffers for standard and sample preparations (Universal Assay Buffer for serum and plasma samples or cell culture medium for cell culture supernatant samples).

Recommended Plate Layout

1	2	3	4	5	6	7	8	9	10	11	12
Standards		**Samples**									
Standard 1	Standard 1	1	1	9	9	17	17	25	25	33	33
Standard 2	Standard 2	2	2	10	10	18	18	26	26	34	34
Standard 3	Standard 3	3	3	11	11	19	19	27	27	35	35
Standard 4	Standard 4	4	4	12	12	20	20	28	28	36	36
Standard 5	Standard 5	5	5	13	13	21	21	29	29	37	37
Standard 6	Standard 6	6	6	14	14	22	22	30	30	38	38
Standard 7	Standard 7	7	7	15	15	23	23	31	31	39	39
Blank	Blank	8	8	16	16	24	24	32	32	40	40

Blank Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												